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We are attempting to identify novel genes in the yeast *S. cerevisiae* that confer gross chromosomal instability (GCI) a hallmark of most breast cancers when deleted. Using a yeast strains carrying the deletion of a unique open reading frame, we will transfect a yeast artificial chromosome (YAC) as a reporter for GCI frequency and determine the quantitative impact of the loss of each gene function. We have constructed the reporter with all of the components for selection and maintenance. The *URA3* reporter functioned correctly during pool growth but the HSV-TK reporter did not. The human BAX genes has been shown to properly function causing cell death in individual and pooled deletion strains. We are modifying the original YAC to incorporate the BAX construct which is superior to the original design. We will use the new YAC on the pool of yeast strains to identify genes affecting GCI and determine the mammalian orthologs of these genes as well as those of interacting partners using *in silico* methods. There is a crucial need to find new candidate genes for breast cancer susceptibility in women and identifying these genes can further improve monitoring and treatment guidelines for women with these mutations.

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Introduction

Our research covered under this grant is to explore a novel approach that can potentially identify breast cancer susceptibility genes that are currently unknown and not deducible from current knowledge. It is based on the fact that most breast cancers exhibit a high degree of genomic instability(1). The increase in the occurrence of gross chromosomal rearrangements is a common theme of most malignant tumors(2). A large proportion of cancer susceptibility syndromes are the result of mutations in genes in DNA repair or in cell-cycle checkpoint pathways in response to DNA damage. Examples include ataxia telangiectasia (AT), Fanconi's anemia (FA), Bloom's syndrome (BS), hereditary non-polyposis colon cancer (HNPCC), Nijmegen breakage syndrome (NBS), and xeroderma pigmentosum (XP). Mutations in such genes, termed "caretaker" genes, are likely to lead to an increased mutation rate of all genes including those directly responsible for cancer, the so-called "gatekeeper" genes(3). Mutations in the caretakers lead to what has been termed a "mutator phenotype" which allows accumulation of the multiple mutations needed to produce cancer(4).

One of the most important DNA lesions that the cell has to repair is the double-strand break (DSB), a lesion that can arise following exposure to genotoxic agents such as ionizing radiation (IR) but also as a consequence of normal cellular processes including DNA replication, meiosis and lymphocyte development(5). Eukaryotic cells have two major pathways for repairing DSB's: Non-homologous end-joining (NHEJ) which rejoins the two broken ends, often with significant loss of sequence, and homologous recombination (HR) which uses the homologous chromosome, or chromatid, as a template to faithfully repair the broken strand. In human cancer it is loss of HR, rather than NHEJ, that is more important in increasing cancer susceptibility (6) (as evidenced by mutations in the ATM, BLM, BRCA-1, BRCA-2, hMRE11, and NBS1 genes, all of which are involved in HR). Although yeast and mammalian cells use both HR and NHEJ to repair DSB's induced by DNA damaging agents, their relative importance is different: Mammalian cells primarily use NHEJ to repair DSB's following IR, whereas yeast primarily use HR. Consequently, mutation of genes affecting HR has a greater impact on radiation sensitivity in yeast than in mammalian cells. A further consequence of the reliance of HR to repair DSB's in yeast is the greater importance of cell-cycle checkpoints in affecting sensitivity to DNA damaging agents in yeast than in mammalian cells. Consequently, yeast (but not mammalian cells) with defects in cell-cycle checkpoints are sensitive to DNA damaging agents, making them excellent models to study defects in these checkpoints.

There is a large degree of homology between genes in yeast and in humans, particularly in the basic cellular processes of DNA repair and cell cycle checkpoints with most of the genes involved being homologous between the two species(7, 8). Consequently, systematically screening yeast for those genes required to minimize gross chromosomal rearrangements will identify those genes in which when mutated in humans fail to maintain genomic fidelity, and hence cancer susceptibility.

Finally, we are working with a powerful new resource in yeast: Namely pools of strains with homozygous deletion of all non-essential genes (9). This has allowed us to identify all genes whose deletion causes sensitivity to UV and other DNA damaging agents(10). The impact of this system and the connection between yeast and cancer is strengthened by our finding that two of the new genes that we identified as producing sensitivity to UV radiation have human homologs that are involved in cancer(8). Using this system to directly determine the genes causing genomic instability in yeast will dramatically advance our understanding of the genetic factors leading to cancer progression.

Body

Hypothesis

The hypothesis to be tested is that novel, human cancer susceptibility genes can be identified by determining novel genes in the budding yeast, *S. cerevisiae*, that confer increases in gross chromosomal instability. As this type of genomic instability is a hallmark of most human cancers and mutations in the corresponding mammalian cells orthologs of yeast genes previously shown to confer genomic instability, also cause genomic instability, we anticipate that our method will identify new genes in mammalian cells, inactivation of which causes genomic instability. We further propose that proteins encoded by these genes that have interactions with known genes involved in DNA repair mechanisms, DNA damage checkpoint functions or fidelity of chromosomal integrity will be candidate breast cancer susceptibility genes and will predispose human cell lines to chromosomal aberrations

Objectives

Our Specific Aims are:
Specific Aim 1: To construct a yeast artificial chromosome which allows for the direct measurement of the occurrence of gross chromosomal rearrangements (YAC-GCR) and transfect it into yeast strains deleted for an individual gene?

Specific Aim 2: To identify novel genes affecting the frequency of gross chromosomal rearrangements in *S. cerevisiae*. Pools of the YAC transected yeast will be grown under non-selective conditions and the rates of spontaneous loss of two individually selectable markers. By hybridization of the DNA from these pools to high density oligonucleotide arrays, we will determine all the genes whose deletion confers increased loss of both markers.

Specific Aim 3: To reconfirm which of the deletion strains in the pooled YAC study also show native chromosomal instability. We will use an established assay for GCR's that we know identifies yeast genes, the human orthologs of which are involved in cancer.

Specific Aim 4: To identify proteins interacting with the proteins encoded by the genes whose deletion produces genomic instability. We will identify interacting proteins by *in silico* methods which exploits the enormous wealth of yeast data of protein-protein interactions identified from systematic yeast two-hybrid studies. Any such proteins which interact with known genes involved in genomic stability or whose homolog is transcriptionally biased in human breast cancer cell lines would go on to further testing.

Specific Aim 5: To determine whether mammalian cells with inhibition of the potential breast cancer susceptibility gene identified in Specific Aims 4 produces genomic instability in the normal human cells in culture. Utilizing the rapidly evolving RNAi technology to selectively reduce the expression of a single gene, we will determine by SKY analysis those candidate genes which demonstrate an increase in chromosomal aberrations.

Results

The specific aims for the first year of the project was to construct a yeast artificial chromosome which allows for the direct measurement of the occurrence of gross chromosomal rearrangements (GCR) and transfect it into yeast strains deleted for an individual gene. We constructed the YAC-GCR as

originally proposed consisting of the *URA3* gene that causes sensitivity to 5-FOA and the *HSV-TK* gene which causes sensitivity to ganciclovir as shown in figure1.

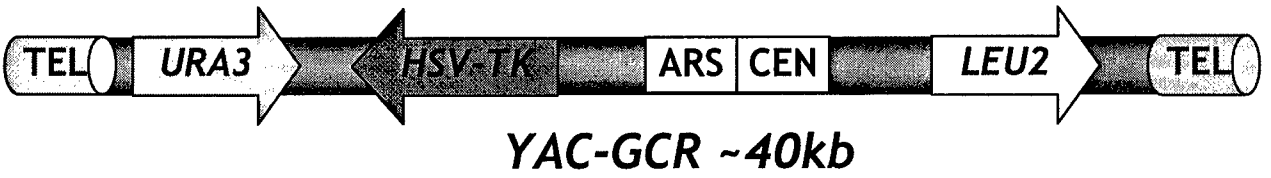

Figure 1: Original design for the yeast artificial chromosome for measuring the rates of gross chromosomal rearrangement in yeast.

In order to determine the dynamic range for this construct, we transfected it into 78 different deletion strains which have a wide spectrum of previously reported GCR frequencies. We tested each strain individually to determine the GCR frequencies derived from the YAC reporter to calibrate these results with a genomically integrated reporter (11). The YAC mediated sensitivity to *URA3* expression in the presence of 5-FOA was highly consistent with previously published results (12). The mutation rates identified with this construct for the resistance to 5-FOA were an order of magnitude higher when compared to the chromosome V inserted *URA3* with an increase linear range making it a very good genome wide screening tool.

The *HSV-TK* reporter was cloned directly from pTK1 (13). In contrast to previous reports (14), the concentrations required to demonstrate any inhibition of cells growth in these strains was greater than 20mg/ml far greater than the 1mg/ml reported. It is likely that the dTMP produced from thymidylate synthetase in this strain was more than sufficient to suppress any incorporation of the toxic analogs produced from ganciclovir by the *HSV-TK* gene product (15). The incorporation of the biosynthetic pathway inhibiting drugs, amethopterin and sulphanilamide, into the protocol to increase the toxicity of HSV-TK expression would have introduced insurmountable difficulties (15). We decided to incorporate a different counter-selectable marker.

Unlike for positive selection, there are very few published counter selection marker genes for yeast. The toxic analog of lysine, alpha-aminoadipate requires that the strain be capable of growth on lysine and that the gene required for its toxicity be located on the YAC. There are several different genes (*LYS2*, *LYS5*, and *LYS14*) that are both capable causing resistance when deleted (16, 17) making this unsuitable for our study. Similarly, the use of methylmercury against the methionine biosynthetic pathway genes presents the same difficulties preventing it use (18). The use of a double *URA3* cassette consisting of two separate and fully functional *URA3* expression constructs each capable of causing sensitivity to 5-FOA was attempted. Through an undetermined mechanism, this construct was toxic to *E. coli*. All of the more than 300 positive clones obtained, contained random deletions inactivating one of the *URA3* genes.

A previous studied identified the over expression of *TPK2* (cAMP-dependent protein kinase) was poisonous to *Saccharomyces* and that this over expression could be regulated by the lack of methionine in the media when controlled via the *MET15* promoter (19, 20). This construct was incorporated into the YAC and tested as a replacement for *HSV-TK*. While the *pMET15-TPK2* construct did inhibit cell growth, it failed to kill the cells allowing for the oscillating of expression of *TPK2* from the *MET15* promoter. The resulting high background of "wildtype" survivors made enumerating the "GCR mutant" survivors problematic. In order to identify a clear delineation between the high GCR and low GCR rates, a cytotoxic marker was required rather than a cytostatic marker which could function in the deletion pool assay.

Recent work by Denko and coworkers expressed the human BAX gene in yeast and demonstrated the induction of apoptotic cell death (21). They used this construct in the yeast deletion

pool and identified all of the genes that were resistant to BAX expression. Only the genes required for the regulated expression from the GAL promoter were resistant to BAX. Due to the problems inherent in galactose induction with the concomitant changes in cellular metabolism and growth rates we sought to modify the expression vector in order to better utilize this gene in the YAC assay. We cloned the *MET15* promoter (20) from genomic DNA as well as the *ADH1* terminator which has been used successfully in many expression vectors (22). In order to identify the correct expression of the BAX protein we also incorporated a FLAG epitope tag for western analysis. The expression plasmid containing the prom*MET15*, FLAG epitope and the term*ADH1* with a useful multiple cloning and restriction sites was constructed for any future expression cassettes which may be required for this or future studies.

We are currently inserting the fully functional BAX cassette into the YAC-GCR backbone creating the new YAC-GCR shown in figure2. With all of the individual components successfully tested for use in deletion pool studies, we anticipate no further delays in the completion of specific aim 1 and rapid progress on specific aim 2.

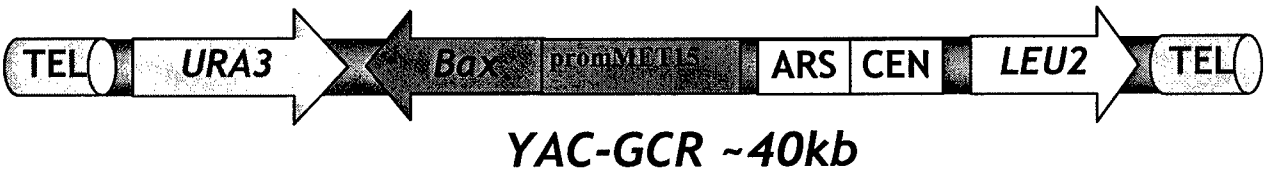

Figure 2. Basic layout of the new yeast artificial chromosomal for measuring the frequency of Gross Chromosomal Rearrangements.

Methods

Specific Aim 1: The construction and transfection of a gross chromosomal rearrangement reporter construct carried on a yeast artificial chromosome (YAC-GCR) will entail basic molecular techniques with readily available components and techniques. The essential components are diagrammed in figure 2. The introduction of two counter-selectable markers will allow for the measurement for chromosomal integrity over a large region. The activity of the *URA3* genes is required for yeast growth in the absence of uracil but imparts sensitivity to the prodrug 5-fluoroorotic acid (5-FOA). The introduction of the Human *BAX* gene under the control of the promoter from the yeast *MET15* gene results in apoptotic cell death when the growth media is lacking in methionine. A gross chromosomal rearrangement event resulting in the spontaneous loss of both of these genes can be measured by the ability to grow in the presence 5-FOA and absence of methionine. The *LEU2* gene will be included on the opposite arm of the YAC allowing the yeast strains carrying the YAC-GCR to grow in media lacking leucine. This allow for the distinction of yeast genes conferring an increase in genomic instability via an increased rate of complete chromosome loss or by genomic rearrangement events while retaining the artificial chromosome backbone. Transfection into diploid yeast strains of the YAC-GCR will be done in 96 well plates of individual homozygous null (non-essential) or heterozygous null (essential genes) yeast deletion strains.

Specific Aim 2: As noted earlier an international consortium has recently generated homozygous strains of *S. cerevisiae* with single gene (open reading frame) knockouts (9). The open reading frame (ORF) knockouts have been made in such a way that individual strains can be identified and their relative representation in a population quantified using standard PCR and complimentary oligonucleotide array technology (Affymetrix, CA). Quantization of each strain in a large mixture of strains using this array technology is possible because each deletion strain has incorporated a unique

molecular "bar code". We have demonstrated both with UV and with other DNA damaging agents that a pool comprising strains of deletion of all non-essential genes can be used to discover the genes whose deletion produces sensitivity to the agents (10, 23).

We will measure the frequency of homozygous deletion strains to spontaneously lose through chromosomal translocation, large internal deletions or de novo telomere additions the activity of two separate marker genes on the YAC-GCR. In addition we will identify those genes which cause a decrease in chromosomal segregation fidelity. We will perform these studies over the first 2 years of the grant.

Specific Aim 3:
Strains that show significant increase in gross chromosomal instability by the hybridization array in specific aim 2 will be tested individually for their sensitivity using the assay developed in Richard Kolodner's laboratory(11, 24). This involves mating a haploid strain deleted for the individual gene with a reporter strain carrying an inserted *URA3* gene at the redundant *HXT13* locus on chromosome V. This gene is located 7.5 kb telomeric to the arginine transporter gene *CAN1* in which no essential genes reside. The resulting diploid strain will be sporulated and haploid strains will be isolated which harbor both the gene deletion and the inserted *URA3* gene. In addition to introducing the selectable marker in the deletion strain, it also insures that the identified instability is due to the gene deletion rather than a spurious secondary mutation in the strain which would segregate separately. The assay involves resistance to both canavanine, a toxic arginine analog, and 5-fluoroorotic acid (5FOA), which has been shown to be the result of loss of the telomeric portion of the chromosome V arm containing the two genes. Kolodner and colleagues have shown that this assay measures gross chromosomal instability, the rate of which is markedly enhanced by deletion of genes whose human orthologs also confer genomic instability in mammalian cells and a predisposition to cancer(25).

Specific Aim 4: We will prioritize candidate genes identified in specific aim 3 by interrogating the vast collection of existing experimental data. Direct sequence comparisons between the yeast and human genomes have identified many primary sequence homologies. Many yeast functional homologs of human genes have been identified which do not score highly in standard primary sequence similarity BLAST searches. They do however; interact with known proteins involved in the same pathways. We will interrogate the large accumulation of data generated from the systematic analysis of the yeast protein interactome achieved by two independent yeast two-hybrid studies(26, 27) and two independent systematic protein complex identification studies by mass spectrometry(28, 29). All candidate genes that show an interaction with proteins having a clear functional human homolog will be studied further. In addition, any candidate gene that has been reported to show a transcriptional bias in breast cancer tissues will be given priority(30, 31).

Specific Aim 5: We will use inhibitory RNA (RNA*i*) to inhibit the proteins encoded by these genes and use a recently described assay involving loss of a chromosomal marker inserted randomly by retroviral transduction into the cells to measure chromosomal instability (Zhang et al., Cancer Research, 2002). This assay involves random chromosomal integration by retrovirus-mediated gene transfer of a selectable marker and tracing this marker through several passages in nonselective medium. The selectable marker will be the hygromycin phosphotransferase (Hyg) gene and herpes simplex virus type I, thymidine kinase gene (TK). Translation of this fusion gene has been shown to confer resistance to hygromycin and sensitivity to (32), thereby providing both positive and negative selectivity. The assay involved measurement of the frequency of gancyclovir-resistant cells (caused by the loss of the functional TK gene following propagation for several generations in nonselective medium). Loss of this marker has been shown to be an indication of chromosomal instability rather

than through point mutations. Genes which, when inhibited, increased genomic instability will be tested further for their effect on chromosomal aberration by SKY analysis.

Key Research Accomplishments

- Constructed a yeast artificial chromosome (YAC) which is capable of stable retention in the yeast deletion mutants as a backbone for further studies.
- Demonstrated the utility of the *URA3* reporter in the YAC as means of measuring the mutational frequency of individual deletion strains in a pool.
- Demonstrated the inadequacies of the HSV-TK mediated ganciclovir toxicity for these studies.
- Cloned the human BAX gene into a methionine regulated expression cassette which will serve as a replacement marker for the failed HSV-TK marker.

Reportable Outcomes:

No reportable outcomes.

Conclusions

With the failure of the HSV-TK reporter construct to adequately discriminate between "mutant" high GCR strains and "wildtype" survivor strains we have been forced to redesign the YAC-GCR. Thorough testing of the individual components, new BAX reporter and the existing *URA3* reporter, has demonstrated the utility of the current design. We are nearing completion of Specific aim 1 and preparing to embark on specific aim 2.

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